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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Assistant Commissioner
for Patents
Washington, D.C. 20231

Dear Sir:

Transmitted herewith for filing is the patent application and oath of the inventor(s): RICHARD L. SMITH

For: SMALL-SCALE HYDROGEN-OXIDIZING-DENITRIFYING BIOREACTOR (SUR-3645)

Date Executed: August 11, 2000

Enclosed are also:

[X] 3 sheet(s) of drawing(s).

Claims as Filed

Claims	Number Filed	Number Extra	Rate X	Basic Fee $690.00
Total Claims	8 - 20	0	$18	= $ -0-
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Please address any correspondence to the undersigned attorney.

Respectfully submitted,

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SMALL-SCALE HYDROGEN-OXIDIZING-DENITRIFYING BIOREACTOR

Field of the Invention

The present invention relates to a method and apparatus for hydrogenating and denitrifying nitrate-contaminated water or waste materials.

Background of the Invention

Nitrate is the most prevalent ground-water contaminant worldwide. Nitrate originates from agricultural, sewage-disposal, and industrial practices from both point and nonpoint sources. Through not exclusive to the subsurface, nitrate contamination is much more pervasive in ground water because nitrate has a relatively long residence time in that environment. Ground water is also the most common drinking water source for both humans and livestock in rural and suburban areas of the United States. Thus, when the nitrate concentration in water from a supply well exceeds drinking water standards (*i.e.*, 10 mg/L nitrogen), the burden typically falls upon the individual user or household to deal with the problem.

The options currently available to treat nitrate contamination on a small scale level are limited. Since nitrate is stable in aqueous solution, it can only be safely removed chemically by techniques such as anion exchange. This can be costly, replaces one salt for another, and at times is ineffective, depending upon the composition of other salts in the water. Moreover, there is the need to dispose of the nitrate that has been removed. Additional, cost-effective

technology to remove nitrate from drinking water is needed: technology that is effective, safe, and practical at the household and livestock supply scales.

Processes for eliminating nitrates from water by denitrification in microbiological reactors are known. These processes, such as those conducted in rising current reactors containing a granular denitrifying biomass, have been described, for example, by Lettings et al., (1980) and by Timmermans, (1983).

For waste waters in particular, different reducing agents such as sugars, less expensive biodegradable organic material, including cellulose and ethanol, have been used. However, only ethanol has been used in treating water that is to be potable. These conventional reducing agents have the disadvantage that they dissolve in water and reduce the quality of the potable water produced. Therefore, it requires another step to eliminate these reducing agents before the water is ready for use.

Verstrate et al., in U.S. Patent No. 4,696,747, describe a process for eliminating nitrates by biological conversion in the presence of hydrogen gas. This process uses alcaligenous eutrophic bacteria, with *Pseudomonas denitrificans* and *Micrococcus denitrificans* being the preferred microorganisms. However, these bacteria cannot grow and remain active in a hydrogen-fed bioreactor when nitrate is not present, particularly when oxygen is removed.

Hydrogen-oxidizing bacteria, some of which are capable of denitrifying nitrogen oxides, are well known and have been studied in detail for many years (Aragno & Schlegel, 1981). Pilot-scale industrial plants that use mixed-culture populations of hydrogen-oxidizing denitrifiers have been operated in Belgium (Liessens et al., 1992) and Germany (Gros et al., 1988) to produce drinking water from nitrate-contaminated ground water. These plants are engineered to produce up to 50 m^3 per day. They are technically complex, require a commercial supply of hydrogen, and trained experts to ensure an adequate function on a daily basis. As a result, an analogous approach or device has not been developed to treat nitrate on a small-scale basis.

Summary of the Invention

It is an object of the present invention to overcome the aforesaid deficiencies of the prior art.

Is is another object of the present invention to provide a bioreactor for treating nitrate-contaminated drinking water.

It is a further object of the present invention to provide a small scale bioreactor for treating nitrate-contaminated drinking water.

It is another object of the present invention to provide a method for treating nitrate-contaminated drinking water even when oxygen is not present in the water being treated.

According to the present invention, autohydrogenotrophic-denitrifying (HOD) bacteria, also known as hydrogen-oxidizing denitrifying bacteria, are used to treat nitrate contamination in water. These bacteria can grow and remain active in a hydrogen-fed bioreactor even when nitrate is not present and even after oxygen has been removed. Of course, there is no reason to attempt to remove nitrate where none is present. However, the function of the bioreactor is much more robust if the bacteria used within it do not need nitrate. For example, the supply of water that is being treated may be shut off for period of time, thus removing the nitrate supply, without affecting the viability of the bacteria within the bioreactor as long as the hydrogen supply is not disrupted. Additionally, some small scale operations may only be used to treat water intermittently. Moreover, these bacteria are more efficient in the exit end of the bioreactor because they do not require a minimal concentration of nitrate to function. Thus, an adequate amount of biomass will be present in the nitrate-free zone of the bioreactor, which helps to insure that the nitrate really is completely removed. This also makes the bioreactor more adaptable to variations in changes in output flow or input nitrate concentration without nitrate breakthrough in the output.

Nitrate-contaminated drinking water is treated with autotrophic, hydrogen-oxidizing denitrifying bacteria which can be isolated from subsurface environments. A low cost

water electrolysis unit that provides a continuous supply of oxygen-free hydrogen is used to generate hydrogen for the process. The bacteria are contained in a flow-through bioreactor which maximizes the ability of the bacteria to remove nitrate in the presence of hydrogen. A sand filtration unit removes unwanted microbial biomass from the treated water.

The present invention provides a small scale nitrate-removal system that uses hydrogen-oxidizing denitrifying bacteria to remove nitrate from the water supplies being used by individual households, farms, or small businesses, the users that are most frequently affected by nitrate contamination and the least likely to find affordable alternative water sources. Flow-through bioreactor systems, *e.g.*, septic tanks, are frequently used on this scale to treat wastewater. The operating parameters for these types of septic systems are also suitable goals for designing a drinking water treatment system. The system of the present invention is cost effective, robust, requires minimal expertise and attention to operate, and produces sufficient quantities of potable water for small scale usage.

The device according to the present invention consists of four principle components:

(1) autotrophic, hydrogen-oxidizing denitrifying (HOD) bacteria isolated from subsurface environments;

(2) a low-cost water electrolysis unit that provides

a continual supply of oxygen-free hydrogen;

(3) a flow-through bioreactor that contains the hydrogen-oxidizing-denitrifying bacteria and is designed to maximize their ability to remove nitrate in the presence of hydrogen; and

(4) a sand filtration unit to remove unwanted microbial biomass from the treated water.

Brief Description of the Drawings

Figure 1 shows the reaction for hydrogen-coupled denitrification using HOD bacteria.

Figure 2 shows a hydrogen generator for use in the present invention.

Figure 3 shows a denitrifying bioreactor and sand filter according to the present invention.

Figure 4 shows nitrate concentrations in the inflow and outflow of a mixed culture bioreactor.

Detailed Description of the Invention

Most current understanding of denitrification as a process, and the denitrifying bacteria themselves, comes from studies relating to nitrogen removal mechanisms in soils and sewage treatment applications. Only recently has the process been studied in more nutrient-poor habitats, such as ground water. These studies have revealed that denitrification can occur in the subsurface under suitable conditions (Smith & Duff, 1988; Spaulding & Parrot, 1994), and that the physical, chemical, and biological factors that control the process in

an aquifer are different from surface soils, sediments, and treated sewage (Brooks et al., 1992; Smith et al., 1992; Smith et al., 1996). The present inventor has also discovered that certain subgroups of denitrifying bacteria, whose ecological role previously had been only poorly studied, can be prominent in ground water. One such group is the hydrogen-oxidizing denitrifiers (Smith et al., 1994).

In the process of isolating and characterizing hydrogen-oxidizing denitrifying bacteria, the present inventor discovered that they are comparatively robust microorganisms that can be used as agents to remediate nitrate-contaminated drinking water on a small scale. The present invention provides a low cost, simple hydrogen delivery system that can be used in conjunction with these microorganisms as a pump and treat approach for nitrate-contaminated waters.

Denitrification is a process mediated by a specialized group of microorganisms. These microbes use nitrate as a respiratory terminal electron acceptor in lieu of oxygen, dissimilating the nitrate to nitrogen gas. Because denitrification is a respiratory process, it can consume relatively large amounts of nitrate, and it produces an innocuous end product. Heterotrophic denitrification has been recognized by the sewage treatment industry for some time as a process that can be manipulated to remove nitrate from treated sewage by adding methanol or some other carbon supply to stimulate denitrifying bacteria. The main limitations of

heterotrophic denitrification, including cost, expertise required, and unwanted by-products which reduce water quality, generally preclude the use of this approach on a small scale basis for treating potable water.

Hydrogen-oxidizing denitrifying (HOD) bacteria obtain their energy by oxidizing hydrogen gas and coupling that to nitrate reduction, as shown in Figure 1. These bacteria occupy a unique ecological niche, one in which there is little competition from other microorganisms. The end products of the HOD process are water and nitrogen gas, which are harmless and inconsequential from the perspective of a drinking water supply, as is the small amount of hydrogen that can dissolve in water. In addition, many of the HOD bacteria in groundwater are autotrophic (Smith et al., 1994). That means that they use carbon dioxide as a carbon source for growth; they have no additional carbon requirements. Because carbon dioxide is present in natural waters as carbonate, these bacteria can be used to remove nitrate in a water supply simply by adding hydrogen gas. This treatment is very selective for HOD bacteria, excluding all other types of microorganisms that could not grow under such conditions. The HOD bacteria can also use hydrogen and respire aerobically. This trait is very useful in a nitrate removal bioreactor because oxygen inhibits denitrification. Thus, oxygen must first be removed from any water supply before denitrification can commence within the reactor. However, the same HOD

culture can effect both oxygen and nitrate removal, as long as an adequate supply of hydrogen is available.

Hydrogen gas has a low solubility in water. This low solubility requires that an excess of hydrogen be always available to remove the quantities of nitrate found in many contaminated water supplies. Hydrogen that is not utilized by HOD bacteria in the treatment process can be easily removed from the water by aeration. Hydrogen can be generated via electrolysis of water, which produces hydrogen gas at the anode and oxygen gas at the cathode at a molar stoichiometry of 2:1. The amount of hydrogen produced is dependent upon the voltage applied to the electrodes and the electrolyte concentration.

Flow-through bioreactors are designed to provide a fixed stationary support for an attached microbial biofilm. The biofilm contacts or is immersed in a flowing aqueous stream and removes or alters the chemical composition of the water via the activity of the attached microorganisms. In some cases, nutrients or substrates for the microorganisms need to be added to the bioreactor. If the substrate is a gas, such as hydrogen, countercurrent flow of the gas and the water is advantageous to increase the availability of the gas to the microorganisms. This can also serve as a mechanism to strip other unwanted gases, such as oxygen, out of solution.

One embodiment of the present invention is shown in Figures 2 and 3, and consists of the following four

components, the numbers within the text referring to the numbered items in the figures:

Component 1. HOD Bacteria

Pure cultures of autotrophic, hydrogen-oxidizing, denitrifying (HOD) bacteria are used as the reactive agents in the flow-through bioreactor used in this invention. The bacteria have been isolated from nitrate-containing groundwater environments. This makes them ideal for such a treatment system because an aquifer is characterized by water flowing through a porous medium, which is identical to the function of the bioreactor. These microorganisms require no organic carbon for growth, only hydrogen, nitrate, and carbon dioxide.

Autohydrogenotrophic (HOD) bacteria are those which obtain energy from the oxidation of molecular hydrogen coupled with the reduction of nitrate to a gaseous form of nitrogen using inorganic carbon as the sole carbon source for cell growth. HOD bacteria are not limited to one single class of microorganism. However, HOD bacteria can be identified by growing the isolate on HOD medium in the presence of hydrogen. Development of turbidity accompanied by loss of nitrate is considered to be a positive result of HOD capacity. This procedure is described in detail in Smith et al., (1994), the entire contents of which are hereby incorporated by reference.

As described in Smith et al., *ibid.*, a number of HOD bacteria were tested and their characteristics identified.

Tables 1 and 2 show characteristics of some of these bacteria and kinetic parameters of hydrogen uptake by some of the cultures of HOD bacteria.

Table 1 Characteristics of hydrogen-oxidizing denitrifying bacteria isolated from nitrate-contaminated groundwater

Strain	Motility	Catalase[a]	Oxidase[a]	Aerobic growth[b] on:												
				Gu	Xy	Me	Su	Fr	Fo	Ci	Ac	Py	Lc	Sc	Gm	Le
HOD 1	+	+	w	–	–	–	–	–	–	–	+	+	+	–	+	–
HOD 2	+	+	+	–	–	–	–	–	–	–	+	+	+	+	+	–
HOD 3	+	w	w	–	–	–	–	–	–	–	+	+	+	–	+	–
HOD 4	+	+	+	–	–	–	–	–	–	–	+	+	+	+	+	–
HOD 5	+	+	w	–	–	–	–	–	–	–	+	+	+	+	+	–
HOD 6	+	+	w	–	–	–	–	–	–	–	+	+	+	+	+	–
HOD 7	–	–	+	+	+	–	+	+	+	+	+	+	+	+	+	+
HOD 8	+	+	+	–	–	–	–	–	–	–	+	+	+	+	+	–
HOD 9	+	+	w	–	–	–	–	–	–	–	+	+	+	+	+	–
P. denitrificans ATCC 17741	–	+	+	+	+	+	+	+	+	–	+	+	+	+	+	+

[a] w, weakly positive.
[b] Substrates tested for growth: Gu, glucose; Xy, xylose; Me, methanol; Su, sucrose; Fr, fructose; Fo, Formate; Ci, citrate; Ac, acetate; Py, pyruvate; Lc, lactate; Sc, succinate; Gm, glutamate; and Le, leucine.

Table 2 Kinetic parameters for hydrogen uptake by cultures of hydrogen-oxidizing denitrifying bacteria with nitrate as the electron acceptor

Strain[a]	K_m (μM)	V_{max} (fmol cell^{-1} h^{-1})
HOD1	0.88	6.14
HOD2	0.70	2.42
HOD3	0.54	2.49
HOD4	1.50	5.24
HOD5	0.30	3.53
HOD6	0.65	3.57
HOD7	3.32	13.29
HOD8[b]	0.38	2.13
	0.79	1.85
	0.71	5.56
HOD9[b]	0.38	2.09
	0.80	1.94
P. denitrificans ATCC 17741	0.77	1.33

[a] Cell growth and uptake assays were done in an autotrophic medium except for HOD 7, for which the medium was supplemented with 3% nutrient broth.
[b] Results from replicate experiments are shown for HOD8 and 9.

In one embodiment of the present invention, Strain HOD5 as described in Tables 1 and 2 was used. This bacterium is a gram negative, motile rod that grows on hydrogen using either oxygen or nitrate as an electron acceptor. It can also grow aerobically on nutrient broth, acetate, pyruvate, lactate, succinate, and glutamate (Table 1). Phylogenetic

analysis of the full sequence of the 16S RNA reveals that HOD 5 belongs to the beta subclass of the *Proteobacteria*, and is most closely related to purple, non-sulfur phototrophic bacteria, particularly *Rhodocyclus* species.

For the bioreactor, a pure culture of HOD 5 is grown in batch culture on hydrogen and nitrate using HOD medium (Smith et al., *ibid*). Following development of turbidity, the culture is transferred to the bioreactor column which has been filled with HOD medium. The culture is grown statically in the bioreactor, with hydrogen flowing, for 2-3 days before the water supply is turned on.

The HOD isolates shown in Table 1 and several other HOD strains isolated from groundwater (Wahlquist, 2000), have been characterized molecularly, the sequence match results are summarized in Table 3. The results shown in the this table are restricted to the top three matches for each isolate, excluding any database strains with sequences less than 1000 base pairs and those that are not aligned to the RDP tree.

Table 3. Summary of Sequence Match results[a]

Isolate	S_{ab}[b]	Full name[c]	Subdivision[d]	Group[e]	Group[e]	Subgroup[e]	Subgroup[e]
#12	0.870	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A[f]	Rcy.tenuis	N/A
	0.867	Rhodocyclus tenuis str. SW18.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.860	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
#27	0.934	Paracoccus denitrificans LMG 4218 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.895	Paracoccus denitrificans DSM 65.	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.895	Paracoccus pantotrophus ATCC 35512 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
#31	0.997	Paracoccus denitrificans DSM 65.	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.997	Paracoccus pantotrophus ATCC 35512 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.993	Paracoccus denitrificans LMG 4218 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
#65	0.986	Paracoccus denitrificans DSM 65.	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.986	Paracoccus pantotrophus ATCC 35512 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
	0.978	Paracoccus denitrificans LMG 4218 (T).	alpha	Rhodobacter-Rhodovulum-Hyphomonas-Rickettsia	Rhodobacter	Paracoccus	Par.denitrificans
#202	0.825	Achromobacter xylosoxidans subsp. denitrificans ATCC 15173 (T).	beta	Bordatella	N/A	Brd.bronchiseptica	N/A
	0.738	Bordetella bronchiseptica str. S-1.	beta	Bordatella	N/A	Brd.bronchiseptica	N/A
	0.711	Bordetella holmesii CDC F5101 (T).	beta	Bordatella	N/A	Brd.bronchiseptica	N/A
#102	0.909	Ochrobactrum anthropi IAM 14119.	alpha	Rhizobium-Agrobacterium	N/A	Brucella Assemblage	N/A
	0.884	Solomonas fluorantheni.	alpha	Rhizobium-Agrobacterium	N/A	Brucella Assemblage	N/A
	0.884	Ochrobactrum anthropi IFO 13694.	alpha	Rhizobium-Agrobacterium	N/A	Brucella Assemblage	N/A
#155	0.738	Ralstonia eutropha str. 335 (R.Y. Stanier) ATCC 17697 (T).	beta	Ral.eutropha	N/A	N/A	N/A
	0.680	Alcaligenes sp. str. M91-3.	beta	Ral.eutropha	N/A	N/A	N/A
	0.660	Ralstonia solanacearum ATCC 11696 (T).	beta	Ral.solanacearum	N/A	Ral.solana	N/A

Table 3, continued.

Isolate	S_{ab}[b]	Full name[c]	Subdivision[d]	Group[e]	Group[e]	Subgroup[e]	Subgroup[e]
#204	0.731	Acidovorax avenae subsp. citrulli ATCC 29625 (T).	beta	Acidovorax	N/A	Acidovorax	Av.avenae
	0.726	Acidovorax avenae subsp. avenae ATCC 19860 (T).	beta	Acidovorax	N/A	Acidovorax	Av.avenae
	0.726	Aquaspirillum psychrophilum str. CA 1 LMG 5408 (T).	beta	Acidovorax	N/A	Acidovorax	Aqsp.psychrophilum
#205	0.749	Aquaspirillum psychrophilum str. CA 1 LMG 5408 (T).	beta	Acidovorax	N/A	Acidovorax	Aqsp.psychrophilum
	0.741	Acidovorax facilis CCUG 2113 (T).	beta	Acidovorax	N/A	Acidovorax	Av.avenae
	0.741	Xylophilus ampelinus ATCC 33914 (T).	beta	Acidovorax	N/A	Acidovorax	Xp.ampelin
#89	0.977	Pseudomonas aeruginosa.	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
	0.975	Pseudomonas aeruginosa LMG 1242 (T).	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
	0.962	Pseudomonas sp. str. CRE 11.	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
#108	0.886	Pseudomonas aeruginosa.	gamma	Pseudomonas and Relatives	N/A	Ps. aeruginosa	N/A
	0.880	Pseudomonas sp. str. CRE 11.	gamma	Pseudomonas and Relatives	N/A	Ps. aeruginosa	N/A
	0.873	Pseudomonas aeruginosa LMG 1242 (T).	gamma	Pseudomonas and Relatives	N/A	Ps. aeruginosa	N/A
#151	0.897	Pseudomonas aeruginosa.	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
	0.881	Pseudomonas sp. str. CRE 11.	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
	0.881	Pseudomonas aeruginosa LMG 1242 (T).	gamma	Pseudomonas and Relatives	N/A	Ps.aeruginosa	N/A
HOD 1[g]	0.760	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.730	Rhodocyclus purpureus str. 6770 DSM 168 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.709	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
HOD 3[g]	0.776	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.719	Rhodocyclus purpureus str. 6770 DSM 168 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.711	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
HOD 4[g]	0.757	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.705	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.705	Rhodocyclus tenuis str. SW18.	beta	Azoarcus	N/A	Rcy.tenuis	N/A

Table 3, continued.

Isolate	S_{ab}[b]	Full name[c]	Subdivision[d]	Group[e]	Group[e]	Subgroup[e]	Subgroup[e]
HOD 5[g]	0.870	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.867	Rhodocyclus tenuis str. SW18.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.860	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
HOD 6[g]	0.774	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.723	Rhodocyclus purpureus str. 6770 DSM 168 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.713	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
HOD 7[g]	0.955	Sinorhizobium fredii LMG 6217 (T).	alpha	Rhizobium-Agrobacterium	N/A	Srh.fredii	N/A
	0.954	Sinorhizobium fredii ATCC 35423 (T).	alpha	Rhizobium-Agrobacterium	N/A	Srh.fredii	N/A
	0.947	Sinorhizobium xinjiangensis IAM 14142.	alpha	Rhizobium-Agrobacterium	N/A	Srh.fredii	N/A
HOD 8[g]	0.775	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.721	Rhodocyclus purpureus str. 6770 DSM 168 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.717	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
HOD 9[g]	0.797	Rhodocyclus tenuis str. 3760 DSM 110.	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.744	Rhodocyclus purpureus str. 6770 DSM 168 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A
	0.740	Rhodocyclus tenuis str. 2761 DSM 109 (T).	beta	Azoarcus	N/A	Rcy.tenuis	N/A

[a]includes the top three RDP Sequence Matches that contain at least 1000 base pairs and have been aligned to the RDP tree
[b]S_{ab} scores range from 0 to 1, with 1 being the closest match possible with a database sequence (see text for complete explanation)
[c]full name of database strain as registered with the RDP (may include accession numbers for culture collections)
[d]based on the tree posted by the RDP; all strains listed belong to subdivisions of the Proteobacteria
[e]phylogenetic groupings on the RDP tree are arranged as a series of nesting hierarchies (e.g., Groups within Groups)
[f]not applicable
[g]Cape Cod isolate of Smith *et al.* (1994)

Sequence Match analyses suggest that those isolates reducing nitrate in the presence of hydrogen in excess of a threshold amount (20% of 1mM) fall into two subdivision of the Proteobacteria. The 16S rRNA gene sequences of isolates 27, 31, and 65 are most similar to those of *Paracoccus denitrificans* strains in the Par. denitrificans subgroups of the Paracoccus subgroup of the Rhodobacter group, which belongs to the alpha subdivision of the Proteobacteria. The sequence of isolate 202 is most similar to that of a strain of *Achromobacter xylosoxidans* subsp. *denitrificans* in the Brd. bronchiseptica subgroup of the Bordatella group, which belongs to the beta subdivision of the Proteobacteria. The 16S rRNA gene sequences of isolates 12, HOD1, HOD3, HOD4, HOD5, HOD6, HOD8, and HOD9 are most similar to those of *Rhodocyclus tenuis* strains in the Rcy. tenuiis subgroup of the Azoarcus group, which belongs to the beta subgroup of the Proteobacteia. The 16S rRNA gene sequence of HOD7 is most similar to strains of *Sinorhizobium fredii* in the Snr. fredii subgroup of the Rhizobium-Agrobacterium group, which belongs to the alpha subdivision of the Proteobacteria.

Sequence match results suggest that those isolates producing less than, but at least 10 percent of, the threshold amount of nitrate reduced in the presence of hydrogen fall into three subdivisions of the Proteobacteria. The 16S rRNA gene sequence of isolate 102 is most similar to that of a strain of *Ochrobactrum anthropi* in the Brucella assemblage of

the Rhizobium-Agrobacterium group, which belongs to the alpha subdivision of the Proteobacteria. The 16S rRNA gene sequence of isolate 155 is most similar to that of a strain of *Ralstonia eutropha* in the Ral. eutropha group, which belongs to the beta subdivision of the Proteobacteria. The 16S rRNA gene sequence of isolate 204 is most similar to that of a strain of *Acidovorax avenae* subsp. *citrulli* in the Av. avenae subgroup of the Acidovorax subgroup of the Acidovorax group, which belongs to the beta subdivision of the Proteobacteria. The 16S rRNA gene sequence of isolate 205 is most similar to that of a strain of *Aquaspirillum psychrophilum* in the Aqsp. psychrophilum subgroup of the Acidovorax subgroup of the Acidovorax group, which belongs to the beta subdivision of the Proteobacteria. The 16S rRNA gene sequences of isolates 89, 108, and 151 are most similar to those of a *Pseudomonas aeruginosa* strain in the Ps. aeruginosa subgroup of the Pseudomonas and relatives group, which belongs to the gamma subdivision of the Proteobacteria.

Table 4 provides raw data from 16S ribosomal RNA gene sequencing.

Table 4
Raw data from 16S ribosomal RNA gene sequencing
A=Adenine, T=Thymine, C=Cytosine, G=Guanine, N=unknown; see Methods section from Wahlquist (2000) for explanation of sequencing method

Isolate #12 full (six-primer) sequence

1	AGAGTTTGAT	CCTGGCTCAG	ATTGAACGCT	GGCGGCATGC	CTTACACATG
51	CAAGTCGAAC	GGCAGCACGG	GAGCTTGCTC	CTGGTGGCGA	GTGGCGAACG
101	GGTGAGTAAT	GCATCGGAAC	GTGCCCTGAA	GTGGGGGATA	ACGCAGCGAA
151	AGTTGCGCTA	ATACCGCATA	TTCTGTGAGC	AGGAAAGCAG	GGGATCGCAA
201	GACCTTGCGC	TTTAGGAGCG	GCCGATGTCG	GATTAGCTAG	TTGGTGGGGT
251	AAAGGCTCAC	CAAGGCGACG	ATCCGTAGCG	GGTCTGAGAG	GATGATCCGC
301	CACACTGGGA	CTGAGACACG	GCCCAGACTC	CTACGGGAGG	CAGCAGTGGG
351	GAATTTTGGA	CAATGGGCGA	AAGCCTGATC	CAGCCATGCC	GCGTGAGTGA
401	AGAAGGCCTT	CGGGTTGTAA	AGCTCTTTCG	GCGGGGAAGA	AATCGCATTC
451	TCTAATACAG	GATGTGGATG	ACGGTACCCG	AATAAGAAGC	ACCGGCTAAC
501	TACGTGCCAG	CAGCCGCGGT	AATACGTAGG	GTGCGAGCGT	TAATCGGAAT
551	TACTGGGCGT	AAAGCGTGCG	CAGGCGGTTT	CGTAAGACAG	ACGTGAAATC
601	CCCGGGCTCA	ACCTGGGAAC	TGCGTTTGTG	ACTGCGAGGC	TAGAGTTTGG
651	CAGAGGGGGG	TGGAATTCCA	CGTGTAGCAG	TGAAATGCGT	AGAGATGTGG
701	AGGAACACCG	ATGGCGAAGG	CAGCCCCCTG	GGCCAATACT	GACGCTCATG
751	CACGAAAGCG	TGGGGAGCAA	ACAGGATTAG	ATACCCTGGT	AGTCCACGCC
801	CTAAACGATG	TCAACTAGGT	GTTGGGAGGG	TTAAACCTCT	TAGTGCCGTA
851	GCTAACGCGT	GAAGTTGACC	GCCTGGGGAG	TACGGCCGCA	AGGCTAAAAC
901	TCAAAGGAAT	TGACGGGGAC	CCGCACAAGC	GGTGGATGAT	GTGGATTAAT
951	TCGATGCAAC	GCGAAAAACC	TTACCTACCC	TTGACATGTC	AGGAATCCCG
1001	GAGAGATTTG	GGAGTGCCCG	AAAGGGAGCC	TGAACACAGG	TGCTGCATGG
1051	CTGTCGTCAG	CTCGTGTCGT	GAGATGTTGG	GTTAAGTCCC	GCAACGAGCG
1101	CAACCCTTGT	CGTTAATTGC	CATCATTCAG	TTGGGCACTT	TAATGAGACT
1151	GCCGGTGACA	AACCGGAGGA	AGGTGGGGAT	GACGTCAAGT	CCTCATGGCC
1201	CTTATGGGTA	GGGCTTCACA	CGTCATACAA	TGGTCGGTCC	AGAGGGTTGC
1251	CAACCCGCGA	GGGGGAGCTA	ATCTCAGAAA	GCCGATCGTA	GTCCGGATTG
1301	CAGTCTGCAA	CTCGACTGCA	TGAAGTCGGA	ATCGCTAGTA	ATCGCGGATC
1351	AGCATGTCGC	GGTGAATACG	TTCCCGGGTC	TTGTACACAC	CGCCCGTCAC
1401	ACCATGGGAG	CGGGTTCTGC	CAGAAGTAGT	TAGCCTAACC	GCAAGGAGGG
1451	CGATTACCAC	GGCAGGGTTC	GTGACTGGGG	TGAAGTCGTA	ACAAGGTAAC
1501	C				

Isolate #27 one-primer (519r) sequence

1	CCGGGGCTTC	TTCTGCTGGT	ACCGTCATTA	TCTTCCCAGC	TGAAAGAGCT
51	TTACAACCCT	AGGGCCTTCA	TCACTCACGC	GGCATGGCTA	GATCAGGGTT
151	GCCCCCATTG	TCTAAGATTC	CCCACTGCTG	CCTCCCGTAG	GAGTCTGGGC
201	CGTGTCTCAG	TCCCAGTGTG	GCTGATCATC	CTCTCAAACC	AGCTATGGAT
251	CGTCGGCTTG	GTAGGCCATT	ACCCCACCAA	CTACCTAATC	CAACGCGGGC
301	TAATCCTTTG	GCGATAAATC	TTTCCCCCGA	AGGGCGCATA	CGGTATTACC
351	CCCAGTTTCC	CAGGACTATT	CCGTACCAAA	GGGCATATTC	CCACGCCGTT
401	ACTCACCCGT	CCGCCGCTCA	CCCCGAAGGG	TGCGCTCGAC	TTGCATGTGT
451	TAGGCCTGCC	GCAGCGTTCG	TTCTGAGCCA	GGATCAAACT	CTGTTGCNCC
501	AATTCGG				

Isolate #31 full (six-primer) sequence

1	AGAGTTTGAT	CCTGGCTCAG	AACGAACGCT	GGCGGCAGGC	CTAACACATG
51	CAAGTCGAGC	GCACCCTTCG	GGTGAGCGG	CGGACGGGTG	AGTAACGCGT
151	GGGAATATGC	CCTTTGGTAC	GGAATAGTCC	TGGGAAACTG	GGGGTAATAC
201	CGTATGCGCC	CTTCGGGGGA	AAGATTTATC	GCCAAAGGAT	TAGCCCGCGT
251	TGGATTAGGT	AGTTGGTGGG	GTAATGGCCT	ACCAAGCCGA	CGATCCATAG
301	CTGGTTTGAG	AGGATGATCA	GCCACACTGG	GACTGAGACA	CGGCCCAGAC
351	TCCTACGGGA	GGCAGCAGTG	GGGAATCTTA	GACAATGGGG	GCAACCCTGA

401 TCTAGCCATG CCGCGTGAGT GATGAAGGCC CTAGGGTTGT AAAGCTCTTT
451 CAGCTGGGAA GATAATGACG GTACCAGCAG AAGAAGCCCC GGCTAACTCC
501 GTGCCAGCAG CCGCGGTAAT ACGGAGGGGG CTAGCGTTGT TCGGAATTAC
551 TGGGCGTAAA GCGCACGTAG GCGGACCGGA AAGTTGGGGG TGAAATCCCG
601 GGGCTCAACC CCGGAACTGC CTTCAAAACT ATCGGTCTGG AGTTCGAGAG
651 AGGTGAGTGG AATTCCGAGT GTAGAGGTGA AATTCGTAGA TATTCGGAGG
701 AACACCAGTG GCGAAGGCGG CTCACTGGCT CGATACTGAC GCTGAGGTGC
751 GAAAGCGTGG GGAGCAAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA
801 AACGATGAAT GCCAGTCGTC GGGCAGCATG CTGTTCGGTG ACACACCTAA
851 CGGATTAAGC ATTCCGCCTG GGGAGTACGG TCGCAAGATT AAAACTCAAA
901 GGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAA
951 GCAACGCGCA GAACCTTACC AACCCTTGAC ATCCCAGGAC CGGCCCGGAG
1001 ACGGGTCTTT CACTTCGGTG ACCTGGAGAC AGGTGCTGCA TGGCTGTCGT
1051 CAGCTCGTGT CGTGAGATGT TCGGTTAAGT CCGGCAACGA GCGCAACCCA
1101 CACTCTTAGT TGCCAGCATT TGGTTGGGCA CTCTAAGAGA ACTGCCGATG
1151 ATAAGTCGGA GGAAGGTGTG GATGACGTCA AGTCCTCATG GCCCTTACGG
1201 GTTGGGCTAC ACACGTGCTA CAATGGTGGT GACAGTGGGT TAATCCCCAA
1251 AAGCCATCTC AGTTCGGATT GGGGTCTGCA ACTCGACCCC ATGAAGTTGG
1301 AATCGCTAGT AATCGCGGAA CAGCATGCCG CGGTGAATAC GTTCCCGGGC
1351 CTTGTACACA CCGCCCGTCA CACCATGGGA GTTGGGTCTA CCCGACGGCC
1401 GTGCGCTAAC CAGCAATGGG GGCAGCGGAC CACGGTAGGC TCAGCGACTG
1451 GGGTGAAGTC GTAACAAGGT AACC

Isolate #65 full (six-primer) sequence

1 AGAGTTTGAT CCTGGCTCAG AACGAACGCT GGCGGCAGGC CTAACACATG
51 CAAGTCGAGC GCACCCTTCG GGGTGAGCGG CGGACGGGTG AGTAACGCGT
101 GGGAATATGC CCTTTGGTAC GGAATAGTCC TGGGAAACTG GGGGTAATAC
151 CGTATGCGCC CTTCGGGGGA AAGATTTATC GCCAAAGGAT TAGCCCGCGT
201 TGGATTAGGT AGTTGGTGGG GTAATGGCCT ACCAAGCCGA CGATCCATAG
251 CTGGTTTGAG AGGATGATCA GCCACACTGG GACTGAGACA CGGCCCAGAC
301 TCCTACGGGA GGCAGCAGTG GGGAATCTTA GACAATGGGG GCAACCCTGA
351 TCTAGCCATG CCGCGTGAGT GATGAAGGCC CTAGGGTTGT AAAGCTCTTT
401 CAGCTGGGAA GATAATGACG GTACCAGCAG AAGAAGCCCC GGCTAACTCC
451 GTGCCAGCAG CCGGCGGTAA TACGGAGGGG GCTAGCGTTG TTCGGAATTA
501 CTGGGCGTAA AGCGCACGTA GGCGGACCGG AAAGTTGGGG GTGAAATCCC
551 GGGGCTCAAC CCCGGAACTG CCTTCAAAAC TATCGGTCTG GAGTTCGAGA
601 GAGGTGAGTG GAATTCCGAG TGTAGAGGTG AAATTCGTAG ATATTCGGAG
651 GAACACCAGT GGCGAAGGCG GCTCACTGGC TCGATACTGA CGCTGAGGTG
701 CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
751 AAACGATGAA TGCCAGTCGT CGGGCAGCAT GCTGTTCGGT GACACACCTA
801 ACGGATTAAG CATTCCGCCT TGGGGAGTAC GGTCGCAAGA TTAAAACTCA
851 AAGGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
901 AAGCAACGCG CAGAACCTTA CCAACCCTTG ACATCCCAGG ACCGGCCCGG
951 AGACGGGTCT TTCACTTCGG TGACCTGGAG ACAGGTGCTG CATGGCTGTC
1001 GTCAGCTCGT GTCGTGAGAT GTTCGGTTAA GTCCGGCAAC GAGCGCAACC
1051 CACACTCTTA GTTGCCAGCA TTTGGTTGGG CACTCTAAGA GAACTGCCGA
1101 TGATAAGTCG GAGGAAGGTG TGGATGACGT CAAGTCCTCA TGGCCCTTAC
1151 GGGTTGGGCT ACACACGTGC TACAATGGTG GTGACAGTGG GTTAATCCCC
1201 AAAAGCCATC TCAGTTCGGA TTGGGGTCTG CAACTCGACC CCATGAAGTT
1251 GGAATCGCTA GTAATCGCGG AACAGCATGC CGCGGTGAAT ACGTTCCCGG
1301 GCCTTGTACA CACCGCCCGT CACACCATGG GAGTTGGGTC TACCCGACGG
1351 CCGTGCGCTA ACCAGCAATG GGGGCAGCGG ACCACGGCTA GGCTCAGCGA
1401 CTGGGGTGAA GTCGTAACAA GGTAACC

Isolate #202 one-primer (519r) sequence

1 GCCGGTGCTA TTCTGCAGGT ACCGTCAGTT CCGCGGGGTA TTAACCCGCG
51 ACGTTTCTTT CCTGCCAAAA GTGCTTTACA ACCCGAAGGC CTTCATCGCA
101 CACGCGGGAT GGCTGGATCA GGGTTTCCCC CATTGTCCAA AATTCCCCAC
151 TGCTGCCTCC CGTAGGAGTC TGGGCCGTGT CTCAGTCCCA GTGTGGCTGG

```
201   TCGTCCTCTC AAACCAGCTA CGGATCGTCG CCTTGGTGAG CCGTTACCCC
251   ACCAACTAGC TAATCCGATA TCGGCCGCTC CAATAGTGCA AGGTCTTGCG
301   ATCCCCTGCT TTCCCCCGTG GGGCGTATGC GGTATTAAGC CACGCTTTCG
351   CGTAGTTATC CCCCGCTACT GGGCACGTTC CGATACATTA CTCACCCGTT
401   CGCCACTCGC CACCAGACCG AAGTCCGTGC TGCCGTCGAC TTGCATGTGT
451   AAGGCATCCC GTAGCGTTAA TCTGAGCCAN GATAAACTCT GTGCGNCAAA
501   NTCGG
```

Isolate #102 one-primer (519r) sequence

```
  1   CGGGGCTTCT TCTCCGGTTA CCGTCATTAT CTTCACCGGT GAAAGAGCTT
 51   TACAACCCTA GGGCCTTCAT CACTCACGCG GCATGGCTGG ATCAGGCTTG
101   CGCCCATTGT CCAATATTCC CCACTGCTGC CTCCCGTAGG AGTCTGGGCC
151   GTGTCTCAGT CCCAGTGTGG CTGATCATCC TCTCAGACCA GCTATGGATC
201   GTCGCTTGGT GAGCCTTTAC CTCACCAACT AGCTAATCCA ACGCGGGCCG
251   ATCCTTTGCC GATAAATCTT TCCCCCGAAG GGCACATACG GTATTAGCAC
301   AAGTTTCCCT GAGTTATTCC GTAGCAAAAG GTACGTTCCC ACGCGTTACT
351   CACCCGTCTG CCGCTCCCCT TGCGGGGCGC TCGACTTGCA TGTGTTAAGC
401   CTGCCGCAGC GTTCGTTCTG AGCCAGGATC AAACTCTGTT GTCNCNAATT
451   CGG
```

Isolate #155 one-primer (519r) sequence

```
  1   CGTAGTTAGC CGGTGCTTAT TCTTCCGGTA CCGTCATCGA CGCCGGGTAT
 51   TAACCAGCGC CATTTCTTTC CGGACAAAAG TGCTTTACAA CCCGAAGGCC
101   TTCTTCACAC ACGCGGCATT GCTGGATCAG GGTTGCCCCC ATTGTCCAAA
151   ATTCCCCACT GCTGCCTCCC GTAGGAGTCT GGGCCGTGTC TCAGTCCCAG
201   TGTGGCTGAT CGTCCTCTCA GACCAGNTAC CTGATCGTCG CCTTGGTAGG
251   CTCTTACCCC ACCAACTAGC TAATCAGACA TCGGCCGCTC CTGTCGCGCG
301   AGGCCGTNAC CGGTCCCNCN CTTTCACNCT CAGGTCGTAT GCGGTATTAA
351   GCTAATCTTT CGACTAGNTA TCCCCCACGA NAGGNCACGT TCCGATGTAT
401   ACTCACNCGT TCGCACTCGC CANCAGGCCG AAGCCCGNNC TGCCGTCNCT
451   TGATGTGAAG GATGCCGCAG CGTTAAC
```

Isolate #204 one-primer (519r) sequence

```
  1   TTCTTACGGT ACCGTCATGA CCCCTCTTTA TTAGAAAGAG GCTTTTCGTT
 51   CCGTACAAAA GCAGTTTACA ACCCGAAGGC CTTCATCCTG CACGCGGCAT
101   GGCTGGATCA GGCTTTCGCC CATTGTCCAA AATTCCCCAC TGCTGCCTCC
151   CGTAGGAGTC TGGGCCGTGT CTCAGTCCCA GTGTGGCTTG ATCATCCTCT
201   CAGACCAGCT ACAGATCGTC GGCTTGGTAA GCTTTTATCC CACCAACTAC
251   CTAATCTGCC ATCGGCCGCT CCGTCCGCGC GAGGTCCGAA GATCCCCCGC
301   TTTCATCCGT AGATCGTATG CGGTATTAGC AAAGCTTTCG CCTCGTTATC
351   CCCCACGATC GGGCACGTTC CGATGTATTA CTACCCGTTC GCACTCGTCA
401   GCATCCGAAG ACCTGGTACC GTNCGACTTG CATGTGTAAG GCATGCCGCA
451   GCGTTAANCT GAGCCNAGGA TCAAACTCTG TTGCGACGA
```

Isolate #205 one-primer (519r) sequence

```
  1   CGGTGCTTAT TCTTACGGTA CCGTCTGACC CCTCTTTATT AGAAAGAGGC
 51   TTTTCGTTCC GTACAAAAGC AGTTTACAAC CCGAAGGCCT TCATCCTGCA
101   CGCGGCATGG CTGGATCAGG CTTTCGCCCA TTGTCCAAAA TTCCCCACTG
151   CTGCCTCCCG TAGGAGTCTG GGCCGTGTCT CAGTCCCAGT GTGGCNTGAT
201   CATCCTCTCA GACCAGCTAC AGATCGTCGG CTTGGTAAGC TTTTATCCCA
251   CCAACTACCT AATCTGCCAT CGGCCGCTCC GTCCGCGCGA GGTCCGAAGA
301   TCCCCCGCTT TCATCCGTAG ATCGTATGCG GTATTAGCAA AGCTNGGGCC
351   TCGTTATCCC CCACGATCGG GCACGTTCCG ATGTATTACT CACCCGTTCG
401   CCACTCGTCA GCATCCGAAG ACCTGTTACC GTTCGACTTG GATGTGTAAG
451   GCATGCCGCA GCGTTCATCT GAGCCANGAT CAACTCTGTG GCGACCAA
```

Isolate #89 full (six-primer) sequence

```
   1  AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCAGGC CTAACACATG
  51  CAAGTCGAGC GGATGAGGGG AGCTTGCTCC TGGATTCAGC GGCGGACGGG
 101  TGAGTAATGC CTAGGAATCT GCCTGGTAGT GGGGGATAAC GTCCGGAAAC
 151  GGGCGCTAAT ACCGCATACG TCCTGAGGGA GAAAGTGGGG GATCTTCGGA
 201  CCTCACGCTA TCAGATGAGC CTAGGTCGGA TTAGCTAGTT GGTGGGGTAA
 251  AGGCCTACCA AGGCGACGAT CCGTAACTGG TCTGAGAGGA TGATCAGTCA
 301  CACTGGAACT GAGACACGGT CCAGACTCCT ACGGGAGGCA GCAGTGGGGA
 351  ATATTGGACA ATGGGCGAAA GCCTGATCCA GCCATGCCGC GTGTGTGAAG
 401  AAGGTCTTCG GATTGTAAAG CACTTTAAGT TGGGAGGAAG GGCAGTAAGT
 451  TAATACCTTG CTGTTTTGAC GTTACCAACA GAATAAGCAC CGGCTAACTT
 501  CGTGCCAGCA GCCGCGGTAA TACGAAGGGT GCAAGCGTTA ATCGGAATTA
 551  CTGGGCGTAA AGCGCGCGTA GGTGGTTCAG CAAGTTGGAT GTGAAATCCC
 601  CGGGCTCAAC CTGGGAACTG CATCCAAAAC TACTGAGCTA GAGTACGGTA
 651  GAGGGTGGTG GAATTTCCTG TGTAGCGGTG AAATGCGTAG ATATAGGAAG
 701  GAACACCAGT GGCGAAGGCG ACCACCTGGA CTGATACTGA CACTGAGGTG
 751  CGAAAGCGTG GGGAGCAAAC AGGATTAGAT ACCCTGGTAG TCCACGCCGT
 801  AAACGATGTC GACTAGCCGT TGGGATCCTT GAGATCTTAG TGGCGCAGCT
 851  AACGCGATAA GTCGACCGCC TGGGGAGTAC GGCCGCAAGG TTAAAACTCA
 901  AATGAATTGA CGGGGGCCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG
 951  AAGCAACGCG AAGAACCTTA CCTGGCCTTG ACATGCTGAG AACTTTCCAG
1001  AGATGGATTG GTGCCTTCGG GAACTCAGAC ACAGGTGCTG CATGGCTGTC
1051  GTCAGCTCGT GTCGTGAGAT GTTGGGTTAA GTCCCGTAAC GAGCGCAACC
1101  CTTGTCCTTA GTTACCAGCA CCTCGGGTGG GCACTCTAAG GAGACTGCCG
1151  GTGACAAACC GGAGGAAGGT GGGGATGACG TCAAGTCATC ATGGCCCTTA
1201  CGGCCAGGGC TACACACGTG CTACAATGGT CGGTACAAAG GGTTGCCAAG
1251  CCGCGAGGTG GAGCTAATCC CATAAAACCG ATCGTAGTCC GGATCGCAGT
1301  CTGCAACTCG ACTGCGTGAA GTCGGAATCG CTAGTAATCG TGAATCAGAA
1351  TGTCACGGTG AATACGTTCC CGGGCCTTGT ACACACCGCC CGTCACACCA
1401  TGGGAGTGGG TTGCTCCAGA AGTAGCTAGT CTAACCGCAA GGGGGACGGT
1451  TACCACGGAG TGATTCATGA CTGGGGTGAA GTCGTAACAA GGTAACC
```

<u>Isolate #108 one-primer (519r) sequence</u>

```
   1  GTCGANTTGC CGGTGCTATT CTGTTGGTAA CGTCAAAAAC AGCAAGGTAT
  51  TAACTTACTG CCCTTCCTCC CAACTTAAAG TGCTTTACAA TCCGAAGACC
 101  TTCTTCACAC ACGCGGCATG GCTGGATCAG GCTTTCGCCC ATTGTCCAAT
 151  ATTCCCCACT GCTGCCTCCC GTAGGAGTCT GGACCGTGTC TCAGTTCCAG
 201  TGTGACTGAT CATCCTCTCA GACCAGTTAC GGATCGTCGC TTGGTAGGCC
 251  TTTACCCCAC CAACTAGCTA ATCCGACCTA GGCTCATCTG ATAGCGTGAG
 301  GTCCGAAGAT CCCCCACTTT CTCCCTCAGG ACGTATGCNN GTATTAGCGC
 351  CCGTTTCCGG ACGTTATCCC CCACTACCAG GCAGATTCCT AGGCATTACT
 401  CACCCGTCCG CCGCTGAATC CAGGAGCAAG CTCCCTTCAT CCGCTCGACT
 451  TGCATGTGTT AGGCCTGCCG CCAGCGTTCA ATCTGAGCCA NGATCAAACT
 501  CTGTTGTCAC GAAATTCGG
```

<u>Isolate #151 one-primer (519r) sequence</u>

```
   1  GTGCTATTCT GTTGGTAACG TCAAAACAGC AAGGTATTAA CTTACTGCCC
  51  TTCCTCCCAA CTTAAAGTGC TTTACAATCC GAAGACCTTC TTCACACACG
 101  CGGCATGGCT GGATCAGGCT TTCGCCCATT GTCCAATATT CCCCACTGCT
 151  GCCTCCCGTA GGAGTCTGGA CCGTGTCTCA GTTCCAGTGT GACTGATCAT
 201  CCTCTCAGAC CAGTTACGGA TCGTCGCTTG GTAGGCCTTT ACCCCACAAC
 251  TAGCTAATCC GACCTAGGCT CATCTGATAG CGTGAGGTCC GAAGATCCCC
 301  CACTTTCTCC CTCAGGACGT ATGCGGTATT AAGCGCCCGT TTCCGGACGT
 351  TATCCCCCAC TACCAGGCAG ATTCCTAGGC ATTACTCACC CGTCCGCCGC
 401  TGAATCCAGG AGCAAGCTCC CTTCATCGCT CGACTTGCAT GTGTTAGGCC
 451  TGCCGCAGCG TTAATCTGAG CCAGGATCAA AC
```

<u>HOD 1 one-primer (519r) sequence</u>

```
   1  TCGTAGTCCG CCGGTGCTTC TTATTCGGGT ACCGTCATCC ACATCCTGTA
```

```
 51   TTAGGAGAAT GCGATTTCTT CCCCGCCGAA AGAGCTTTAC AACCCGAAGG
101   CCTTCTTCAC TCACGCGGCA TGGCTGGATC AGGCTTTCGC CCATTGTCCA
151   AAATTCCCCA CTGCTGCCTC CCGTAGGAGT CTGGGCCGTG TCTCAGTCCC
201   AGTGTGGCGG ATCATCCTCT CAGACCCGCT ACGGATCGTC GCCTTGGTGA
251   GCCTTTACCC CACCAACTAG CTAATCCGAC ATCGGCCGCT CCTAAAGCGC
301   AAGGTCTTGC GANCCCCTGC TTTCCTGCTC ACAGAATATG CGGTATTAGC
351   GCAACTTTCG CTGCGTTATC CCCCACTTCA GGGCACGTTC CGATGCATTA
401   CTCACCCGTT CGCCACTCGC CACCAGGAGC AAGCTCCCGT GCTGCCGTTC
451   GACTTGCATG TGTAAGGCAT GCCGCCAGCG TTCAATCTGA GCCAGGATCA
501   AACTCTGTTG TCACGAAATT CGG
```

HOD 3 one-primer (519r) sequence

```
  1   AGTNGCCGGT GCTTCTTATT CGGGTACCGT CATCCACATC CTGTATTAGA
 51   GAATGCGATT TCTTCCCCGC CGAAAGAGCT TTACAACCCG AAGGCCTTCT
101   TCACTCACGC GGCATGGCTG GATCAGGCTT TCGCCCATTG TCCAAAATTC
151   CCCACTGCTG CCTCCCGTAG GAGTCTGGGC CGTGTCTCAG TCCCAGTGTG
201   GCGGATCATC CTCTCAGACC CGCTACGGAT CGTCGCTTGG TGAGCCTTTA
251   CCCCACCAAC TAGCTAATCC GACATCGGCC GCTCCTAAAG CGCAAGGTCT
301   TGCGATCCCC TGCTTTCCTG CTCACAGAAT ATGCGGTATT AAGCGCAACT
351   TTCGCTTGCG TTATCCCCCA CTTCAGGGCA CGTTCCGATG CATTACTCAC
401   CCGTTCGCCA CTCGCCACCA GGAGCAAGCT CCCGTGCTGC CGTTCGACTT
451   GCATGTGTAA GGCATGCCGC CAGCGTTCAA TCTGAGCCAN GATCAAACTC
501   TGTTGTCACG NAAATTCGG
```

HOD 4 one-primer (519r) sequence

```
  1   AGTNCGCCGG TGCTTCTTAT TCGGGTACCG TCATCCACAT CCTGTATTAN
 51   GAGAATGCGA TTTCTTCCCC GCCGAAAGAG CTTTACAACC CGAAGGCCTT
101   CTTCACTCAC GCGGCATGGC TGGATCAGGC TTTCGCCCAT TGTCCAAAAT
151   TCCCCACTGC TGCCTCCCGT AGGAGTCTGG GCCGTGTCTC AGTCCCAGTG
201   TGGCGGATCA TCCTCTCAGA CCCGCTACGG ATCGTCGCCT TGGTGAGCCT
251   TTACCCCACC AACTAGCTAA TCCGACATCG GCCGCTCCTA AAGCGCAAGG
301   TCTTGCGATC CCCTGCTTTC CTGCTCACAG AATATGCGGT ATTAGCGCAA
351   CTTTCGCTTG CGTTATCCCC CACTTCAGGG CACGTTCCGA TGCATTACTG
401   ACCCGTTCGC CACTCGCCAC CAGGAGCAAG CTCCCGTGCT GCCGTTCGAC
451   TTGCATGTGT AAGGCATGCC GCCAGNGTTC AATCTGAGCC ANGATCAAAC
501   TCTGTTGTCA CGAATTCGGN NNNNC
```

HOD 5 full (six-primer) sequence

```
  1   AGAGTTTGAT CCTGGCTCAG ATTGAACGCT GGCGGCATGC CTTACACATG
 51   CAAGTCGAAC GGCAGCACGG GAGCTTGCTC CTGGTGGCGA GTGGCGAACG
101   GGTGAGTAAT GCATCGGAAC GTGCCCTGAA GTGGGGGATA ACGCAGCGAA
151   AGTTGCGCTA ATACCGCATA TTCTGTGAGC AGGAAAGCAG GGGATCGCAA
201   GACCTTGCGC TTTAGGAGCG GCCGATGTCG GATTAGCTAG TTGGTGGGGT
251   AAAGGCTCAC CAAGGCGACG ATCCGTAGCG GGTCTGAGAG GATGATCCGC
301   CACACTGGGA CTGAGACACG GCCCAGACTC CTACGGGAGG CAGCAGTGGG
351   GAATTTTGGA CAATGGGCGA AAGCCTGATC CAGCCATGCC GCGTGAGTGA
401   AGAAGGCCTT CGGGTTGTAA AGCTCTTTCG GCGGGGAAGA AATCGCATTC
451   TCTAATACAG GATGTGGATG ACGGTACCCG AATAAGAAGC ACCGGCTAAC
501   TACGTGCCAG CAGCCGCGGT AATACGTAGG GTGCGAGCGT TAATCGGAAT
551   TACTGGGCGT AAAGCGTGCG CAGGCGGTTT CGTAAGACAG ACGTGAAATC
601   CCCGGGCTCA ACCTGGGAAC TGCGTTTGTG ACTGCGAGGC TAGAGTTTGG
651   CAGAGGGGGG TGGAATTCCA CGTGTAGCAG TGAAATGCGT AGAGATGTGG
701   AGGAACACCG ATGGCGAAGG CAGCCCCCTG GGCCAATACT GACGCTCATG
751   CACGAAAGCG TGGGGAGCAA ACAGGATTAG ATACCCTGGT AGTCCACGCC
801   CTAAACGATG TCAACTAGGT GTTGGGAGGG TTAAACCTCT TAGTGCCGTA
851   GCTAACGCGT GAAGTTGACC GCCTGGGGAG TACGGCCGCA AGGCTAAAAC
901   TCAAAGGAAT TGACGGGGAC CCGCACAAGC GGTGGATGAT GTGGATTAAT
951   TCGATGCAAC GCGAAAAACC TTACCTACCC TTGACATGTC AGGAATCCCG
```

1001	GAGAGATTTG	GGAGTGCCCG	AAAGGGAGCC	TGAACACAGG	TGCTGCATGG
1051	CTGTCGTCAG	CTCGTGTCGT	GAGATGTTGG	GTTAAGTCCC	GCAACGAGCG
1101	CAACCCTTGT	CGTTAATTGC	CATCATTCAG	TTGGGCACTT	TAATGAGACT
1151	GCCGGTGACA	AACCGGAGGA	AGGTGGGGAT	GACGTCAAGT	CCTCATGGCC
1201	CTTATGGGTA	GGGCTTCACA	CGTCATACAA	TGGTCGGTCC	AGAGGGTTGC
1251	CAACCCGCGA	GGGGGAGCTA	ATCTCAGAAA	GCCGATCGTA	GTCCGGATTG
1301	CAGTCTGCAA	CTCGACTGCA	TGAAGTCGGA	ATCGCTAGTA	ATCGCGGATC
1351	AGCATGTCGC	GGTGAATACG	TTCCCGGGTC	TTGTACACAC	CGCCCGTCAC
1401	ACCATGGGAG	CGGGTTCTGC	CAGAAGTAGT	TAGCCTAACC	GCAAGGAGGG
1451	CGATTACCAC	GGCAGGGTTC	GTGACTGGGG	TGAAGTCGTA	ACAAGGTAAC
1501	C				

<u>HOD 6 one-primer (519r) sequence</u>

1	GNCGTAGTTA	GCCGGTGCTT	CTTATTCGGG	TACCGTCATC	CACATCCTGT
51	ATTANGAGAA	TGCGATTTCT	TCCCCGCCGA	AAGAGCTTTA	CAACCCGAAG
101	GCCTTCTTCA	CTCACGCGGC	ATGGCTGGAT	CAGGCTTTCG	CCCATTGTCC
151	AAAATTCCCC	ACTGCTGCCT	CCCGTAGGAG	TCTGGGCCGT	GTCTCAGTCC
201	CAGTGTGGCG	GATCATCCTC	TCAGACCCGN	TACGGATCGT	CGCCTTGGTG
251	AGCCTTTACC	CCACCAACTA	GCTAATCCGA	CATCGGCCGC	TCCTAAAGCG
301	CAAGGTCTTG	CGATCCCCTG	CTTTCCTGCT	CACAGAATAT	GCGGGTATTA
351	AGCGCAACTT	TCGCTGCGTT	ATCCCCCACT	TCAGGGCACG	TTCCGATGCA
401	TTACTCACCC	GTTCGCCACT	CGCCACCAGG	AGCAAGCTCC	CGTGCTGCCG
451	TTCGACTTGC	ATGTGTAAGG	CATGCCGCCA	GCGTTCAATC	TGAGCCAGGA
501	TCAAACTCTG	TTGTCACGAA	AC		

<u>HOD 7 full (six-primer) sequence</u>

1	AGAGTTTGAT	CCTGGCTCAG	AACGAACGCT	GGCGGCAGGC	TTAACACATG
51	CAAGTCGAGC	GCCCCGCAAG	GGGAGCGGCA	GACGGGTGAG	TAACGCGTGG
101	GAATCTACCC	TTTTCTACGG	AATAACGCAG	GGAAACTTGT	GCTAATACCG
151	TATACGCCCT	TCGGGGGAAA	GATTTATCGG	GAAAGGATGA	GCCCGCGTTG
201	GATTAGCTAG	TTGGTGGGGT	AAAGGCCTAC	CAAGGCGACG	ATCCATAGCT
251	GGTCTGAGAG	GATGATCAGC	CACATTGGGA	CTGAGACACG	GCCCAAACTC
301	CTACGGGAGG	CAGCAGTGGG	GAATATTGGA	CAATGGGCGC	AAGCCTGATC
351	CAGCCATGCC	GCGTGAGTGA	TGAAGGCCCT	AGGGTTGTAA	AGCTCTTTCA
401	CCGGTGAAGA	TAATGACGGT	AACCGGAGAA	GAAGCCCCGG	CTAACTTCGT
451	GCCAGCAGCC	GCGGTAATAC	GAAGGGGGCT	AGCGTTGTTC	GGAATTCTGG
501	GCGTAAAGCG	CACGTAGGCG	GACATTTAAG	TCAGGGGTGA	AATCCCGGGG
551	CTCAACCCCG	GAACTGCCTT	TGATACTGGG	TGTCTAGAGT	ATGGAAGAGG
601	TGAGTGGAAT	TCCGAGTGTA	GAGGTGAAAT	TCGTAGATAT	TCGGAGGAAC
651	ACCAGTGGCG	AAGGCGGCTC	ACTGGTCCAT	TACTGACGCT	GAGGTGCGAA
701	AGCGTGGGGA	GCAAACAGGA	TTAGATACCC	TGGTAGTCCA	CGCCGTAAAC
751	GATGAATGTT	AGCCGTCGGG	CAGTTTACTG	TTCGGTGGCG	CAGCTAACGC
801	ATTAAACATT	CCGCCTGGGG	AGTACGGTCG	CAAGATTAAA	ACTCAAAGGA
851	ATTGACGGGG	GCCCGCACAA	GCGGTGGAGC	ATGTGGTTTA	ATTCGAAGCA
901	ACGCGCAGAA	CCTTACCAGC	CCTTGACATC	CCGATCGCGG	ATTACGGAGA
951	CGTTTTCCTT	CAGTTCGGCT	GGATCGGAGA	CAGGTGCTGC	ATGGCTGTCG
1001	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG	TCCCGCAACG	AGCGCAACCC
1051	TCGCCCTTAG	TTGCCAGCAT	TTAGTTGGGC	ACTCTAAGGG	GACTGCCGGT
1101	GATAAGCCGA	GAGGAAGGTG	GGGATGACGT	CAAGTCCTCA	TGGCCCTTAC
1151	GGGCTGGGCT	ACACACGTGC	TACAATGGTG	GTGACAGTGG	GCAGCGAGAC
1201	CGCGAGGTCG	AGCTAATCTC	CAAAAGCCAT	CTCAGTTCGG	ATTGCACTCT
1251	GCAACTCGAG	TGCATGAAGT	TGGAATCGCT	AGTAATCGCA	GATCAGCATG
1301	CTGCGGTGAA	TACGTTCCCG	GGCCTTGTAC	ACACCGCCCG	TCACACCATG
1351	GGAGTTGGTT	CTACCCGAAG	GTAGTGCGCT	AACCGCAAGG	AGGCAGCTAA
1401	CCACGGTAGG	GTCAAGCGAC	TGGGGTGAAG	TCGTAACAAG	GTAACC

<u>HOD 8 one-primer (519r) sequence</u>

1	GTCGTAGTTG	CCGGTGCTTC	TTATTCGGGT	ACCGTCATCC	ACATCCTGTA

51 TTANGAGAAT GCGATTTCTT CCCCGCCGAA AGAGCTTTAC AACCCGAAGG
101 CCTTCTTCAC TCACGCGGCA TGGCTGGATC AGGCTTTCGC CCATTGTCCA
151 AAATTCCCCA CTGCTGCCTC CCGTAGGAGT CTGGGCCGTG TCTCAGTCCC
201 AGTGTGGCGG ATCATCCTCT CAGACCCGCT ACNGGATCGT CGCCTTGGTG
251 AGCCTTTACC CCACCAACTA GCTAATCCGA CATCGGCCGC TCCTAAAGCG
301 CAAGGTCTTG CGATCCCCTG CTTTCCTGCT CACAGAATAT GCGGTATTAG
351 CGCAACTTTC GCTTGCGTTA TCCCCCACTT CAGGGCACGT TCCGATGCAT
401 TACTCACCCG TTCGCCACTC GCCACCAGGA GCAAGCTCCC GTGCTGCCGT
451 TCGACTTGCA TGTGTAAGGC ATGCCGCAGC GTTCAATCTG AGCCANGATC
501 AAACTCTGTT GTCAC

HOD 9 one-primer (519r) sequence

1 GNCGTAGTTA GCCGGTGCTT CTTATTCGGG TACCGTCATC CACATCCTGT
51 ATTANGAGAA TGCGATTTCT TCCCCGCCGA AAGAGCTTTA CAACCCGAAG
101 GCCTTCTTCA CTCACGCGGC ATGGCTGGAT CAGGCTTTCG CCCATTGTCC
151 AAAATTCCCC ACTGCTGCCT CCCGTAGGAG TCTGGGCCGT GTCTCAGTCC
201 CAGTGTGGCG GATCATCCTC TCAGACCCGC TACNGGATCG TCGCCTTGGT
251 GAGCCTTTAC CCCACCAACT AGCTAATCCG ACATCGGCCG CTCCTAAAGC
301 GCAAGGTCTT GCGATCCCCT GCTTTCCTGC TCACAGAATA TGCGGTATTA
351 GCGCAACTTT CGCTGCGTTA TCCCCCACTT CAGGGCACGT TCCGATGCAT
401 TACTCACCCG TTCGCCACTC GCCACCAGGA GCAAGCTCCC GTGCTGCCGT
451 TCGACTTGCA TGTGTAAGGC ATGCCGCCAG CGTTCAATCT GAGCCANGAT
501 CAAACTCTGT TGTCACNAAA AC

Heterotophic denitrifiers have been isolated from nearly every environment and are extraordinarily diverse, including thermophiles, diazotrophs, psychrophiles, halophiles, budding bacteria, gliding bacteria, pathogens, phototrophs, fermentative bacteria, magnetotactic bacteria, and others. They are distributed among the division of the domains Archaea and Bacteria. In the Bacteria they include Gram-positive organisms (*e.g.*, actinomycetes, mycobacteria, *Bacillus*) and Gram-negative organisms (*e.g.*, agrobacteria, pseudomonads, *Neisseria, Cytophaga, Aquifex, Campylobacter*).

The four identified autohydrogenotrophic denitrifying bacteria reported in the literature belong to the Proteobacteria division of the domain Bacteria. The Proteobacteria consist of the Gram-negative purple photosynthetic bacteria and their nonphotosynthetic relatives. The division is exceptionally diverse and is divided into five subdivisions: the alpha subdivision (*e.g.*, purple nonsulfur bacteria, rhizobacteria, agrobacteria, *Nitrobacter*), the beta subdivision (*e.g.*, *Alcaligenes, Rhodocyclus, Bordatella, Neisseria, Thiobacillus*), the gamma subdivision (*e.g.*, purple sulfur bacteria, *Azobacter, Chromatium*, Enterobacteriaceae, the pseudomonads, *Vibrio*), the delta subdivision (e.g., mycobacteria, *Bdellovibrio*, *Desulfovibrio*) and the epsilon subdivision (*e.g., Campylobacter, Wolinella*).

Based on this information, it does not appear that the autohydrogenotrophic denitrifying bacteria would form a

monophyletic group. However, one skilled in the art can, without undue experimentation, readily determine if a microorganism is an HOD bacterium by testing it as described above. That is, by growing an isolate on HOD medium as described above in the presence of hydrogen, development of turbidity accompanied by loss of nitrate is considered to be a positive result of HOD capacity.

Component 2. Hydrogen Genrator

The use of hydrogen-enhanced denitrification to remove nitrate from a water supply ultimately depends upon the availabilty of a low-cost, continual source of hydrogen gas. While electrolytic hydrogen generators are currently rather expensive, other means can be used to produce hydrogen for denitrification of water by this method. Other techniques for generating hydrogen gas include corrosive oxidation of Fe(0) or basalt that produces cathodic hydrogen gas from water, biological fermentation or electrolysis units that can operate with a low voltage power supply.

In one embodiment of this invention, hydrogen gas is produced by hydrolysis of water in a dual-chamber, glass reservoir (2). The two chambers are each sealed with a pressure-tight screw top cap that is penetrated with a platinum wire electrode (3). The chambers are connected via hollow glass tubing and contain 4 N sodium hydroxide. The rate of hydrogen gas evolution in the hydrogen generator is dependent upon the concentration of sodium hydroxide used in

the hydrogen generator. Therefore, the sodium hydroxide concentration can be adjusted to match the amount of hydrogen required for a specific bioreactor application. Potassium hydroxide can be used as a substitute for the sodium hydroxide.

A 12 volt 2 amp DC electrical potential is continuously applied to the electrodes using a commercial automobile battery charger (1). Oxygen gas is produced in the cathode chamber and is channeled via metal tubing through a sodium hydroxide trap (5) to an adjustable gas flow controller (6). Hydrogen gas is produced in the anode chamber and is channeled through a sodium hydroxide trap (5), a check valve (7) to prevent back flow, and into the bioreactor (8-10). Internal pressure within the chambers of the hydrogen generator is balanced using the adjustable flow controller.

Component 3 Flow-through Bioreactor

The flow-through bioreactor (8-10) is constructed from plastic pipe and fitted with sealed endcaps. The bioreactor is filled with a coarse porous medium (9) such as washed pea gravel (2-4 mm in diameter) or plastic or glass beads, which serve as solid surfaces to support biofilm formation by the HOD bacteria. Nitrate-laden water is pumped into the top of the reactor and travels downward through the porous medium where it contacts the microbial biofilm, and exits out the bottom of the bioreactor nitrate-free. The water level within the bioreactor is controlled by the height

of the exit tube.

Hydrogen gas enters the bioreactor via an airstone (10) in the bottom. Hydrogen bubbles travel upward, countercurrent to water flow, and are vented out the top endcap. In addition to serving as a substrate for the HOD bacteria, the hydrogen bubbles strip oxygen from the influent water and nitrogen gas from water within the reactor that is produced *via* the denitrification reaction. The headspace volume in the bioreactor is designed not to exceed 1-5% of the total volume of the bioreactor to minimize the amount of hydrogen gas present within the system.

Component 4. Sand Filtration Unit.

The nitrate-free water exiting the bioreactor then percolates *via* gravity flow through a sand filtration unit (11-13). This unit is constructed with pipe, generally made of plastic, fitted with a bottom endcap. The unit is filled with a bottom layer of coarse porous medium such as pea gravel 4-6 inches thick, and overlain with clean, coarse to-medium grained sand (12). On top of the sand column is a block (13) to evenly distribute the input water over the surface of the sand. The overall height of the sand filter unit is approximately equivalent to the height of the water column within the bioreactor. In the sand filter, the water is aerated and filtered to remove suspended microorganisms from the bioreactor effluent. The top layer of sand within the

infiltration unit is periodically removed and replaced with clean sand. Water exits the sand filter unit via a tube inserted in the bottom endcap.

Preferred and Extreme Ranges of Conditions

For water with a nitrate concentration of about 2 mM (28 mg/L nitrogen), the optimum hydraulic residence time in the bioreactor is about 1.5-2 hours at a temperature of 25°C. The bioreactor can effectively remove nitrate concentrations of about 0.7 to 20 mM (10-280 mg/L nitrogen) in a pH range of about 6-9.

A bioreactor as described above was grown initially with HOD medium and then switched to well water input. The water used had a total dissolved solids load of 204 mg/l, an alkalinity of 190 mg/l as $CaCO_3$, and a pH of 8. This was selected to test the bioreactor using a water source that would represent a challenge for the HOD bacteria, given the composition and pH of the well water. The well water was used "as is", except that nitrate was added. No effort was made to provide nutrients required for HOD growth, such as trace minerals, phosphorus, or inorganic carbon, or to remove indigenous ground-water bacteria. In general, the mixed-culture bioreactor was able to remove nitrate from the well-water input; nitrate levels in the output were well below the drinking water limit, as shown in Figure 4. There were several instances when the output nitrate concentrations were high, but these were all due to an inadvertent shutdown of the ~~hydrogen generator. It was discovered that routine~~

hydrogen generator. It was discovered that routine

NOTE: missing TEXT

ADDED 8/11/2000 Richard Smith

replacement of the water consumed by hydrolysis within the hydrogen generator was important. After 100 days of operation, the nitrate concentration in the input was significantly increased, without any appreciable effect upon the function of the bioreactor (Figure 4).

The device of the present invention provides for small-scale treatment of nitrate-contaminated water. The process and apparatus of the present invention provide for the complete removal and destruction of nitrate from a water supply. The apparatus is small scale and cost effective. The device has its own hydrogen generator, and uses specially chosen autotrophic, hydrogen-oxidizing-denitrifying bacteria that have been isolated from ground water environments. The water filtration unit is low cost and low maintenance.

The apparatus of the present invention comprises four principle components: (1) autotrophic, hydrogen-oxidizing denitrifying bacteria isolated from subsurface environments; (2) a low-cost water electrolysis unit that provides a continual supply of oxygen-free hydrogen; (3) a flow-through bioreactor that contains the HOD bacteria and is designed to maximize their ability to remove nitrate in the presence of hydrogen; and (4) a filtration unit to remove unwanted microbial biomass from the treated water. The present invention provides an important new combination of components to treat nitrate-contaminated water on a small scale basis. Of particular importance is the use of purple, non-sulfur

phototrophic bacteria to treat nitrate contamination in combination with hydrogen.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying current knowledge, readily modify and/or adapt for various applications such specific embodiments without departing from the generic concept, and, therefore, such adaptions and modifications should and are intended to be comprehended within the meaning and range of equivalents of the disclosed embodiments. It is to be understood that the phraseology or terminology employed herein is for the purpose of description and not of limitation.

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WHAT IS CLAIMED IS:

1. A method for treating nitrate-contaminated water comprising treating said water with autotrophic, hydrogen-oxidizing denitrifying bacteria in the presence of hydrogen.

2. The method according to claim 1 wherein the bacteria are purple, non-sulfur phototrophic bacteria.

3. The method according to claim 1 wherein the hydrogen is produced by hydrolysis of water.

4. The method according to claim 1 wherein the bacteria have been isolated from nitrate-containing groundwater.

5. An apparatus for treating nitrate-contaminated water comprising:

(a) a pure culture of autotrophic, hydrogen-oxidizing denitrifying bacteria;

(b) a hydrogen generator;

(c) a flow-through bioreactor; and

(d) a filtration unit.

6. The apparatus of claim 5 wherein said hydrogen generator comprises a dual-chamber reservoir wherein each chamber is sealed with a pressure-tight cap penetrated with an electrode, the chambers connected by hollow tubing and containing a solution of sodium hydroxide or potassium hydroxide.

7. The apparatus of claim 5 wherein the flow-through bioreactor is filled with a porous medium for supporting biofilm formation by the bacteria.

8. The apparatus of claim 5 wherein the filtration unit comprises a sand filtration unit.

ABSTRACT OF THE DISCLOSURE

A method for treating nitrate-contaminated water comprising treating said water with hydrogen-oxidizing denitrifying bacteria in the presence of hydrogen. The apparatus for use in this method preferably comprises :

(a) a pure culture of autotrophic, hydrogen-oxidizing denitrifying bacteria;

(b) a hydrogen generator;

(c) a flow-through bioreactor; and

(d) a filtration unit.

f:\...\usge\smith9\pro\specification

$$H_2 + NO_3^- \xrightarrow{\text{HOD BACTERIA}} H_2O + N_2$$

$$CO_2 \longrightarrow \text{CELL CARBON}$$

FIGURE 1. HYDROGEN COUPLED DENITRIFICATION

Fig 2. Hydrogen Generator

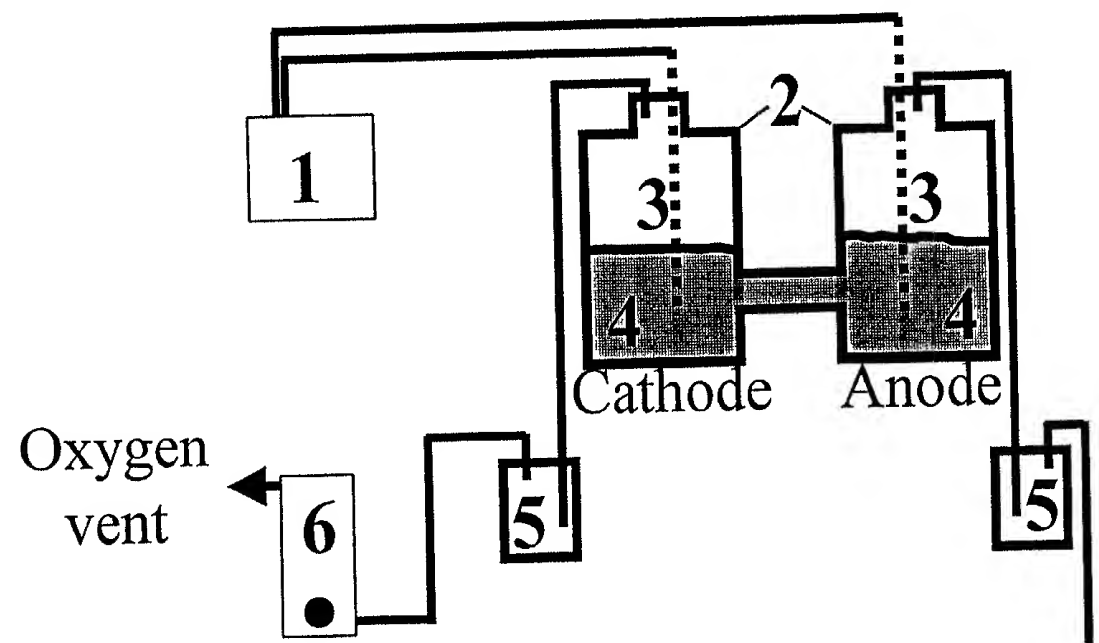
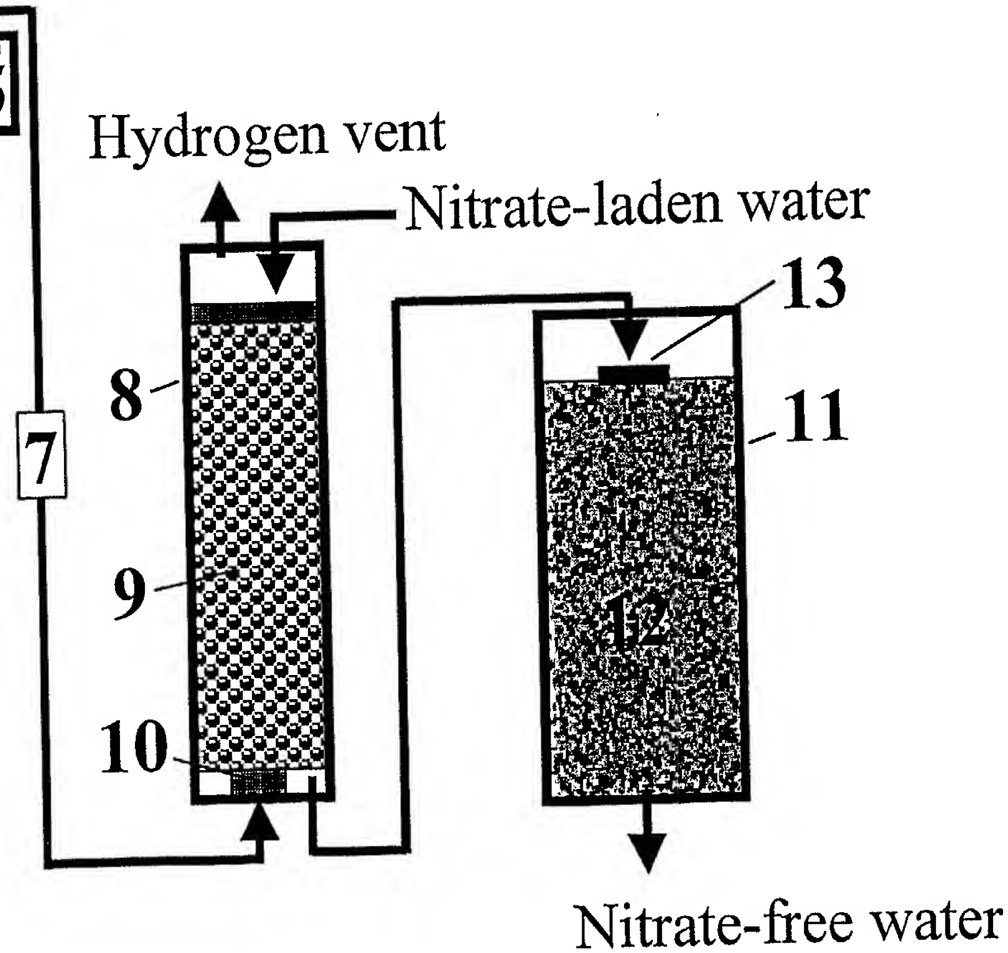


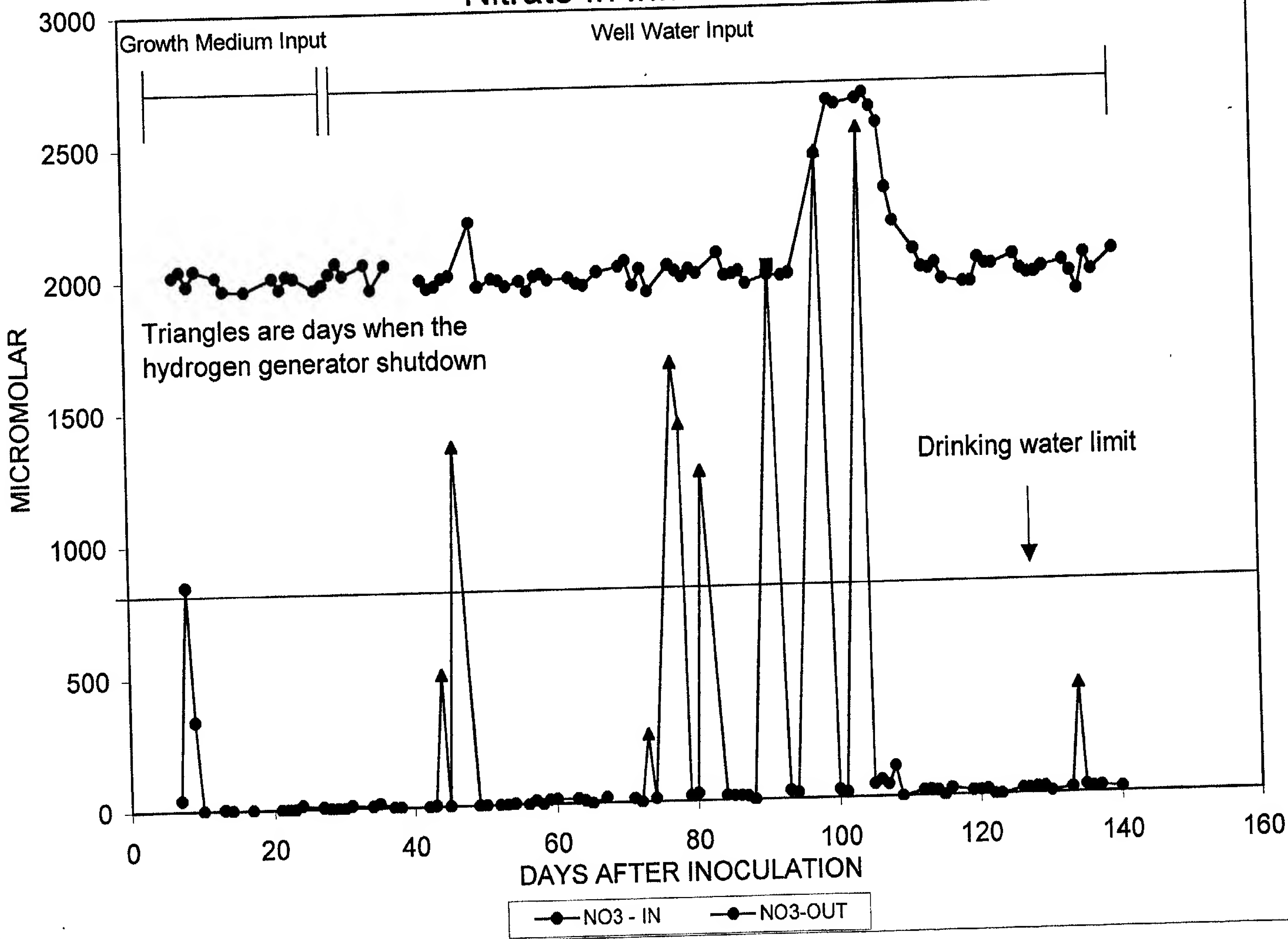
Fig 3. Denitrifying Bioreactor and Sand Filter



Numbered Items

1. 12 volt, 2 amp, DC power supply
2. Glass containers, with pressure tight screw top lids, connected via side arm tubing
3. Platinum wire electrodes
4. 4 N Sodium hydroxide
5. Sodium hydroxide trap
6. Adjustable flow meter
7. Check valve
8. Flow-through plastic pipe, with endcaps
9. Sorted pea-gravel, 2-4 mm
10. Airstone
11. Plastic pipe with endcap on bottom
12. Washed sand
13. Water distribution block

Figure 4. Mixed Culture-Bioreactor
Nitrate In Inflow & Outflow



DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION
English Language Declaration

As below named inventors, we hereby declare that:

Our residences, post office addresses, and citizenships are as stated below next to our names.

We believe we are the original, first, and joint inventors of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SMALL-SCALE HYDROGEN-OXIDIZING-DENITRIFYING BIOREACTOR
(SUR-3645)

the specification of which (check one):

[X] is attached hereto.

[ ] Was filed on ______________________________ as

Application Serial No. ______________________________

and was amended on ______________________________
(if applicable)

We hereby state that we have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment specifically referred to in the oath or declaration.

We acknowledge the duty to disclose information which is material to the examination of this application in accordance with Title 37, Code of Federal Regulations, § 1.56(a).

We hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate listed below and have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

Prior Foreign Application(s)

			Priority Claimed	
(Number)	(Country)	(Day/Month/Year Filed)	YES	NO
(Number)	(Country)	(Day/Month/Year Filed)	YES	NO
(Number)	(Country)	(Day/Month/Year Filed)	YES	NO

We hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, we acknowledge the duty to disclose material information as defined in Title 37, Code of Federal Regulations, § 1.56(a) which occurred between the filing date of the prior application and the national or PCT international filing date of this application:

Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

We hereby declare that all statements made herein of our own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As named inventors, we hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office in connection therewith.

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